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TITLE: The role of glucocorticoids and neuroinflammation in mediating the effects of stress on drug abuse

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14. ABSTRACT Drug abuse and misuse is a major health hazard in the military as well as in the population more generally. There have been major recent advances in our understanding of the alterations in the brain produced by drugs of abuse, and in how the "addicted brain" differs from the normal brain. However, many individuals who experience, or are exposed to a drug of abuse do not develop addiction, or abuse the drug. For example, the overwhelming majority of patients that receive opiates for pain relief while hospitalized do not develop opiate addiction. That is, use does not always develop into abuse, and the factors that mediate this transition are largely unknown. The development of an understanding of the factors and brain mechanisms that throw the balance towards the development of abuse from use would be a major step in the development of therapies that can ameliorate addiction. The core hypothesis is that stressors, via their production of increased glucocorticoids (GCs), sensitize microglia so that these cells produce excessively high levels of inflammatory mediators such as IL-1 when acted upon by drugs of abuse, and that this process is responsible, in whole or in part, for the increased vulnerability to drug abuse produced by stressful experiences. This is a novel, and previously unexplored hypothesis. The work, if successful, could lead to a re-conceptualization of GCs as a <i>sensitization factor</i> that induces a vulnerability to neuroinflammatory processes and thereby open a new field of investigation into the role of stress and GCs in the etiology of substance abuse disorders.					
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Introduction

Drug abuse and misuse is a major health hazard in the military as well as in the population more generally. There have been major recent advances in our understanding of the alterations in the brain produced by drugs of abuse, and in how the “addicted brain” differs from the normal brain. However, many individuals who experience, or are exposed to a drug of abuse neither develop addiction, nor abuse the drug. For example, the overwhelming majority of patients that receive opiates for pain relief while hospitalized do not develop opiate addiction. That is, use does not always develop into abuse, and the factors that mediate this transition are largely unknown. The development of an understanding of the factors and brain mechanisms that throw the balance towards the development of abuse from use would be a major step in the development of therapies that can ameliorate addiction.

Drug abuse is exacerbated by deployment in war zones, and particularly by exposure to trauma, resulting in high co-morbidity with PTSD. Although there is a wealth of human and animal data clearly demonstrating that exposure to stressful conditions potentiates drug taking, the development of addiction, and the reinstatement of extinguished drug self-administration, the mechanisms involved are poorly understood, and consequently there are few, if any, preventative or curative treatments. The hallmark of the stress response is an increase in adrenal glucocorticoids (GCs) (cortisol in the human, corticosterone in the rodent), and it is known that the GC response to stressors is involved in the facilitation of addictive processes produced by stress. Thus, for example, adrenalectomy prevents stressor-induced potentiation of drug self-administration, as well as the augmentation of drug-induced dopamine release in reward-related areas of the brain produced by prior stress. However, the mechanism(s) by which stress and GCs exaggerate behavioral and neurochemical responses to drugs of abuse are poorly understood, and so therapeutic targets have correspondingly not been identified. The goals of the present proposal are to further our understanding of how stress and/or GCs potentiate responses to drugs of abuse, and to identify therapeutic targets that would allow the blockade of stress effects on drug use and addiction.

In the second year of this project, we made pivotal discoveries in how stress sensitizes the pro-inflammatory effects of methamphetamine (METH) in brain reward pathways as well as showing that stress potentiates self-administration of METH. It should be noted that METH became a focal point for our studies in light of evidence showing that stress failed to potentiate the pro-inflammatory effects of cocaine and failed to potentiate the self-administration of morphine.

Body

Specific Aim I. Do stress and/or glucocorticoids potentiate neuroinflammatory responses to drugs of abuse?

IA. Acute stress and acute rises in glucocorticoids.

1A1. Stress.

In the first year of this project, we reported that transcriptional profiling of the Nucleus accumbens (NAcc), prefrontal cortex (PFC), and ventral tegmental area (VTA) showed that stress potentiated the neuroinflammatory response to METH in NAcc (IL-1b), VTA (TNFa), and PFC (IL-1b) 2h post-METH treatment. We have now confirmed these effects at the protein level.

Animals were exposed to a single session of acute stress (100, 1.0 mA, 5 s tailshocks delivered via fixed electrodes while restrained in Plexiglas tubes, the standard acute stressor used in our laboratory), or serve as home cage controls (HCC). 24 hours after stressor exposure, animals were treated with METH (10 mg/kg ip) or vehicle (0.9% saline). 2 hours after drug or vehicle treatment, whole brain was flash frozen in liquid nitrogen and tissue micropunches of brain reward pathways (NAcc, PFC and VTA) were made. Protein from tissue micropunches was analyzed for IL-1b and TNFa.

1A1. Results

Effect of acute stress on the IL-1b protein response to METH

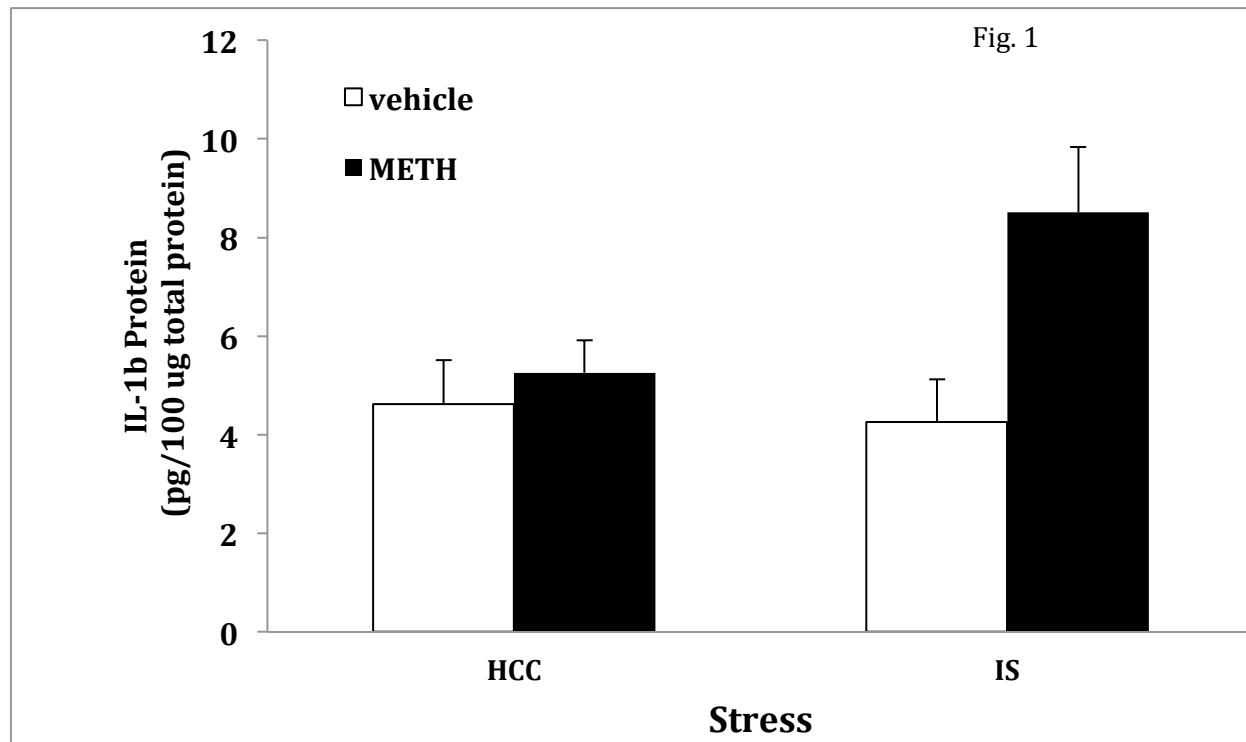


Fig. 1 is representative data from PFC showing stress-induced potentiation of the IL-1b response to METH. The observed protein effects show a stress x METH interaction on IL-1b protein levels, which confirms our observations of a similar interaction at the mRNA level. The protein and mRNA data clearly show that exposure to severe acute stress potentiates the neuroinflammatory response to METH. While there is a considerable literature showing that METH is neuroinflammatory (Clark et al., 2013), the data presented here is the first demonstration that prior exposure to a stressor potentiates the neuroinflammatory response to METH. These data support our main hypothesis that severe acute stress sensitizes the neuroinflammatory response to METH. We have previously reported that severe acute stress sensitizes the neuroinflammatory response to pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), which is a pro-inflammatory component of the cell-wall of gram negative bacteria (e.g. *E. coli*)(Johnson et al., 2003). PAMPs function as danger signals to

the innate immune system. The data presented here suggests that METH may also be functioning as a danger signal to microglia, which are the predominant innate immune effector cells in the CNS. Microglia are considered sentinels of the CNS that perform the function of immunosurveillance for danger (Ransohoff & Cardona, 2010). The present data suggested that severe acute stress may sensitize microglia to the direct pro-inflammatory effects of METH in vitro, which prompted us to explore this hypothesis under Specific Aim IIA1.

Specific Aim IB. Repeated stress and repeated rises in glucocorticoids.

This aim has not been addressed. Studies addressing **specific aims IB1. Repeated stress, IB2. Repeated corticosterone and IB3. Duration** will commence in January of 2014.

Specific Aim IC. Glucocorticoid mediation

This aim has not been addressed. Studies addressing specific aims **IC1. Acute stress and IC2. Repeated stress** will commence in April of 2014. In light of our findings in IA1., only the neuroinflammatory effects of METH will be tested in Aim IB and IC.

Specific Aim II. Do microglia mediate the effects of stress and glucocorticoids on neuroinflammatory responses to drugs of abuse?

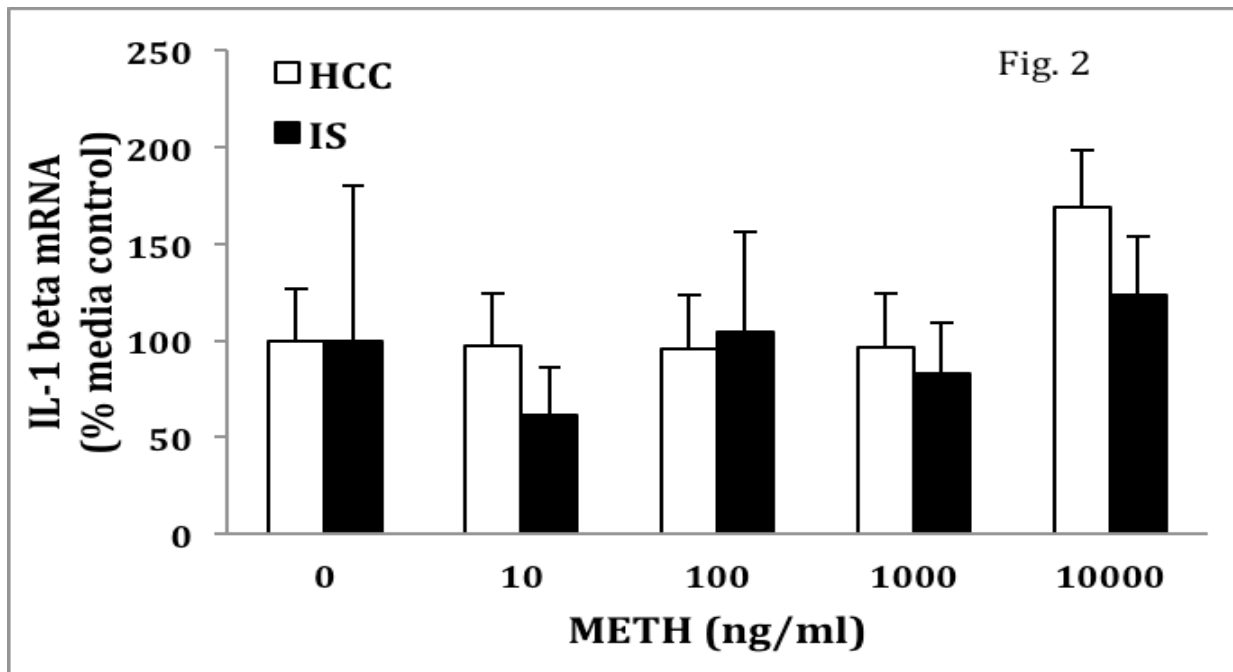
IIA. Microglial sensitization.

IIA1. Basic effect

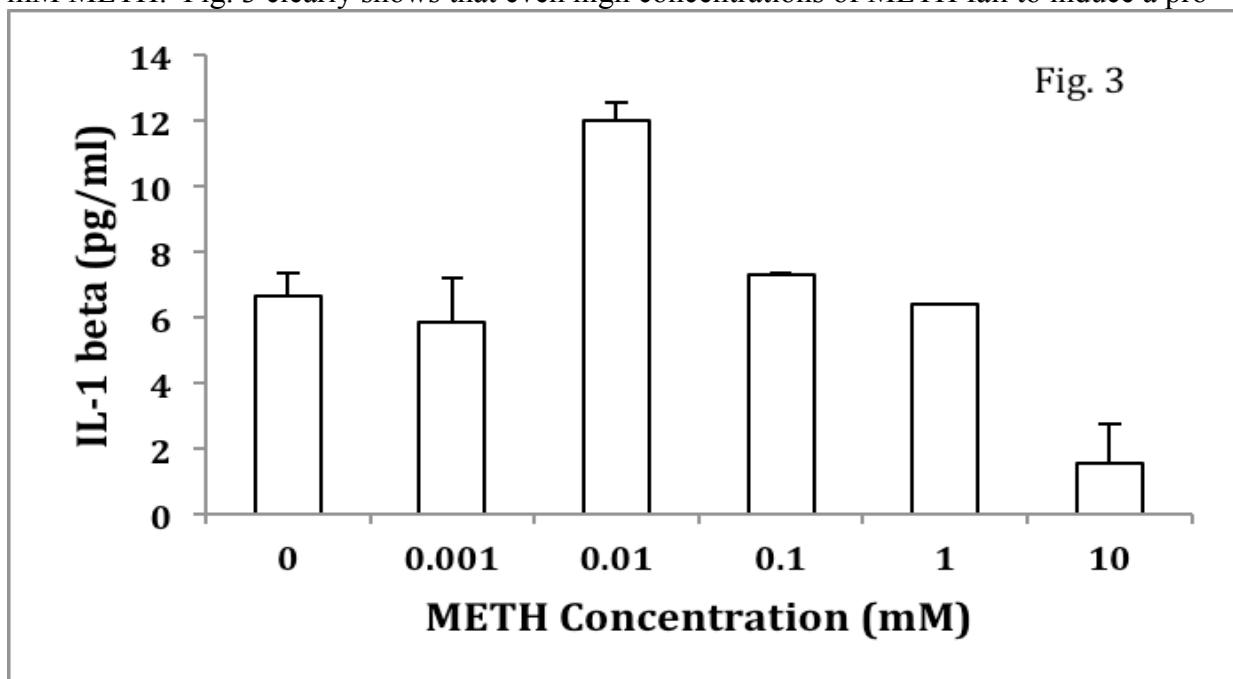
In this experiment, animals were exposed to a single session of acute stress (100, 1.0 mA, 5 s tailshocks delivered via fixed electrodes while restrained in Plexiglas tubes, the standard acute stressor used in our laboratory), or serve as home cage controls (HCC). 24 hr post-stress exposure, microglia were isolated from whole striatum. It should be noted that we were unable to isolate sufficient numbers of microglia from tissue micropunches to conduct in vitro experiments and thus we utilized whole striatum, which encompasses the NAcc, as our source of microglia. Microglia were isolated using a Percoll density gradient as previously described in a prior publication from our laboratory (Frank et al., 2006). Microglia were suspended in DMEM + 10% FBS media and plated at 5×10^3 /well in a 96-well microtiter plate. Microglia were exposed to METH (0, 10, 100, 1000 and 10,000 ng/ml) for 24 hr. Supernatants were collected for IL-1b protein analysis and cells collected for analysis of IL-1b mRNA.

IIA1. Results

Contrary to our hypothesis, severe acute stress failed to potentiate the pro-inflammatory response of microglia to METH ex vivo (Fig. 2). Fig. 2 shows the IL-1b mRNA response to stress and METH. Similar results were obtained with IL-1b protein. Interestingly, METH, independent of stress condition, failed to induce a pro-inflammatory response significantly greater than media control. This latter finding is not consistent with prior studies showing that METH can induce a pro-inflammatory response in microglia cell lines (Tocharus et al., 2010). However, the effects of METH in primary microglial cells, as used here, has not been reported. One possible explanation for the null effects of METH found here may have been due to insufficient concentrations of drug. To examine this possibility, we conducted several follow-up studies to



determine if higher concentrations of METH would induce a pro-inflammatory response in isolated striatal microglia. In this experiment, striatal microglia were isolated from non-stressed animals and exposed to METH (0, 0.001, 0.01, 0.1, 1, and 10 mM) for 24 hr. Cell supernatants were analyzed for IL-1b protein levels and cells analyzed for IL-1b mRNA levels. Consistent with the data presented in Fig. 2, METH failed to induce a pro-inflammatory response even at a concentration of 10 mM (Fig. 3). Fig. 3 shows IL-1b protein levels in supernatant. It should be noted that Fig. 2 presents METH concentrations as ng/ml and in Fig. 3 METH concentrations are presented as molarity. For the purpose of comparison, 10,000 ng/ml METH is equivalent to 0.03 mM METH. Fig. 3 clearly shows that even high concentrations of METH fail to induce a pro-



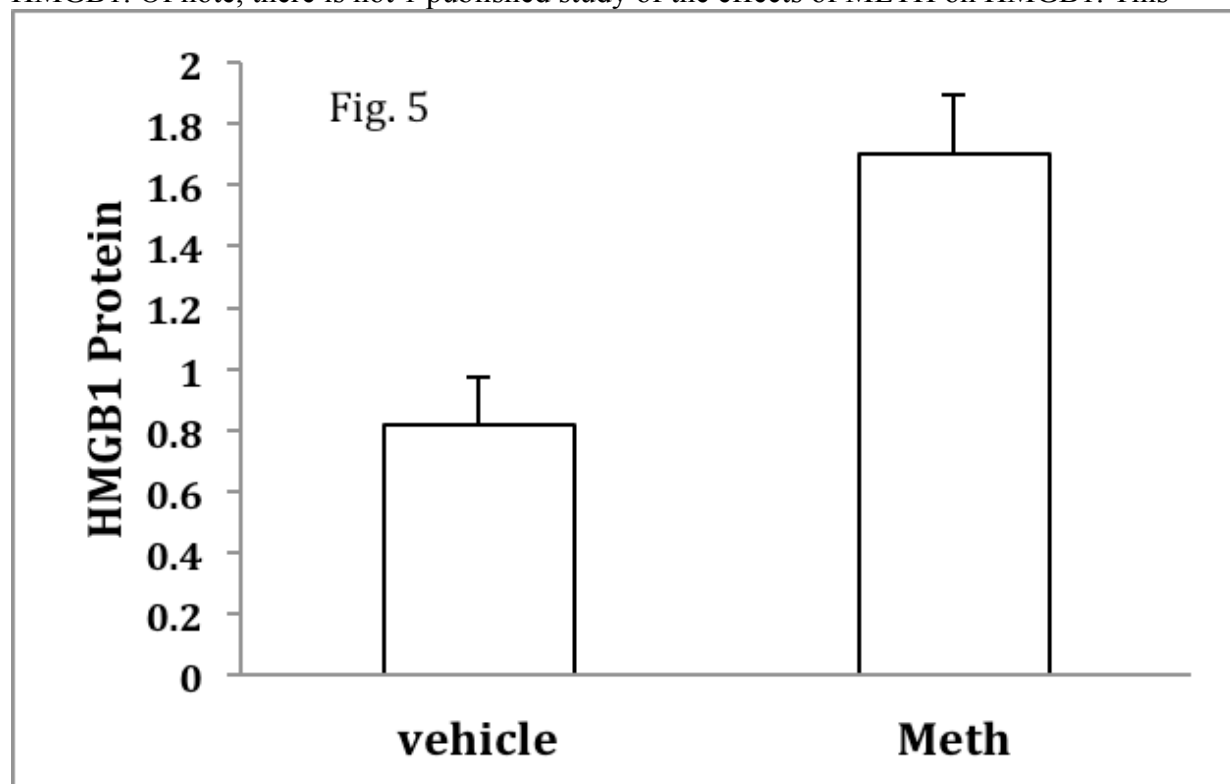
inflammatory response in microglia. In addition, we found that METH failed to affect cell viability (data not shown). These data are the first to show that in primary microglia METH is not directly inflammatory. Prior studies examined the effects of METH in microglia cells lines (Tocharus et al., 2010), which may account for the discrepancy between our findings and prior findings.

These findings clearly preempt addressing further questions posed under Specific Aim II. Therefore, specific aims **IIA2. Glucocorticoid mediation, IIB1a. Acute stress, IIB1b. Repeated stress, and IIB2. TLR blockade** will not be pursued because these aims necessitate that METH directly induce a pro-inflammatory response in microglia. However, the null findings presented in Fig. 2 and Fig. 3 suggest that since METH does not directly induce a pro-inflammatory response in microglia, METH may be inducing a pro-inflammatory response in vivo through an indirect pathway in the CNS. That is, the neurotoxic effects of METH may result in the release of a danger signal in the CNS, which then induces a pro-inflammatory response in microglia. A considerable body of evidence shows that METH is neurotoxic resulting in neuronal damage and death (Krasnova & Cadet, 2009). In addition, METH induces an array of toxic mediators such as reactive oxygen species, which can be pro-inflammatory. As a proxy for Specific Aim II, we are proposing to explore a mechanism whereby METH is pro-inflammatory in vivo and testing whether stress potentiates this pro-inflammatory mechanism. As an experimental strategy to target a specific pro-inflammatory mediator of METH effects in vivo, we started by asking whether there is a pro-inflammatory mediator or danger signal induced by severe acute stress that may be involved in neurotoxic responses. One intriguing possibility is the alarmin, high mobility group box-1 (HMGB1) protein

Briefly, HMGB1 is an endogenous molecule that is found in the nucleus of most cell types. It is passively released by necrotic, but not apoptotic cells. In addition, HMGB1 is actively secreted by monocytes, macrophages, & dendritic cells in response to PAMPs and stimulation by IL-1b. HMGB1 was first thought to signal through the receptor for advanced glycation end products (RAGE). However, recent work indicates that HMGB1 also signals through TLR2 and TLR4. Indeed, TLR 2 and 4 appear to be especially critical in the mediation of HMGB1 effects. Very recent reports suggest that HMGB1 stimulation of TLRs requires HMGB1 to first complex with other molecules such as IL-1, but this does not change its basic role. HMGB1 action on immune cells produces the full array of pro-inflammatory effects—immune cells are attracted, PICs, ROS, NO, and PGs are release (Yanai et al., 2012).

We are proposing to explore the notion that stress potentiates the neurotoxic effects of METH in a two-step process. First, stress induces the release of HMGB1, which sensitizes microglia to subsequent pro-inflammatory stimuli. Second, when a stress-sensitized animal is subsequently exposed to METH, METH induces neuronal damage in dopaminergic neurons resulting in the release of additional HMGB1, which then targets TLR2 and TLR4 receptors on microglia. HMGB1 ligation of these receptors on sensitized microglia results in a potentiated pro-inflammatory response. We have begun addressing the question of whether HMGB1 mediates the stress-induced potentiation of the neuroinflammatory response to METH. In recent studies using severe acute stress, we have found that stress induces microglia to release HMGB1. Animals were exposed to our standard acute stress protocol and immediately after termination of the stressor, microglia were isolated and cultured for 24 hr. Fig. 4 shows HMGB1 protein levels in supernatants of microglia from naive and stressed animals. Acute stress induced microglia to release greater amounts of HMGB1 protein. We have also found that stress increases the expression of HMGB1 in CNS whole tissue (data not shown). A recent study published from our

lab showed that pharmacological blockade of TLR2 and TLR4 receptors in the CNS significantly attenuated the stress-induced sensitization of microglia (Weber et al., 2013). It should be noted that the study of Weber et al. (2013) was not funded by the present DOD project, but the data is highly relevant to the present project. The findings of Weber et al. suggests that HMGB1 may be mediating stress-induced sensitization effects given that HMGB1 primes innate immune cells through TLR2 and TLR4. In light of these effects of stress on HMGB1, we then addressed the question of whether METH also induces HMGB1 in the CNS. In this experiment, non-stressed, drug-naive animals were injected with METH (10 mg/kg ip) or vehicle and 2 hr post-injection, striatum was dissected and HMGB1 protein levels measured by Western blot. The dose and timing of METH exposure used here is identical to the parameters of METH exposure used in our prior experiments. Fig. 5 shows that METH induces a nearly 2-fold increase in striatal HMGB1. Of note, there is not 1 published study of the effects of METH on HMGB1. This



finding could provide fundamental insights into how METH induces a pro-inflammatory immune response in the CNS. The data presented in Fig. 4 and 5 provide a strong evidentiary basis to examine whether HMGB1 mediates stress-induced potentiation of the neuroinflammatory response to METH. As a first step towards addressing this hypothesis, we are currently undertaking a set of experiments to characterize METH-induced upregulation of HMGB1 in brain reward nuclei including the NAcc, VTA, and PFC. A subsequent set of experiments will address whether prior exposure to acute stress potentiates the METH-induced HMGB1 response in these nuclei. It should be noted that these experiments do not change our SOW, but simply involves assessing a new analyte (HMGB1 protein) in **Aim 1A1. Stress**. If the data from these experiments support our hypothesis, we will conduct a set of experiments that will involve blockade of HMGB1 signaling using a neutralizing antibody. In a related project not

funded under the present proposal, we have collected preliminary evidence that blockade of HMGB1 signaling during stress exposure ameliorates stress-induced sensitization of microglia. It should be noted that we currently are not conducting studies using this alternate drug (anti-HMGB1 neutralizing antibody) under the present proposal. However, if experiments show that stress potentiates METH-induced HMGB1 increases in brain reward nuclei, we will, of course, submit a revised SOW as well as an ACURO prior to conducting studies using anti-HMGB1 to test whether METH-induced HMGB1 mediates METH-induced neuroinflammatory effects.

Specific Aim III. Do microglial activation and consequent neuroinflammatory responses mediate the effects of stress and glucocorticoids on drug abuse behavior?

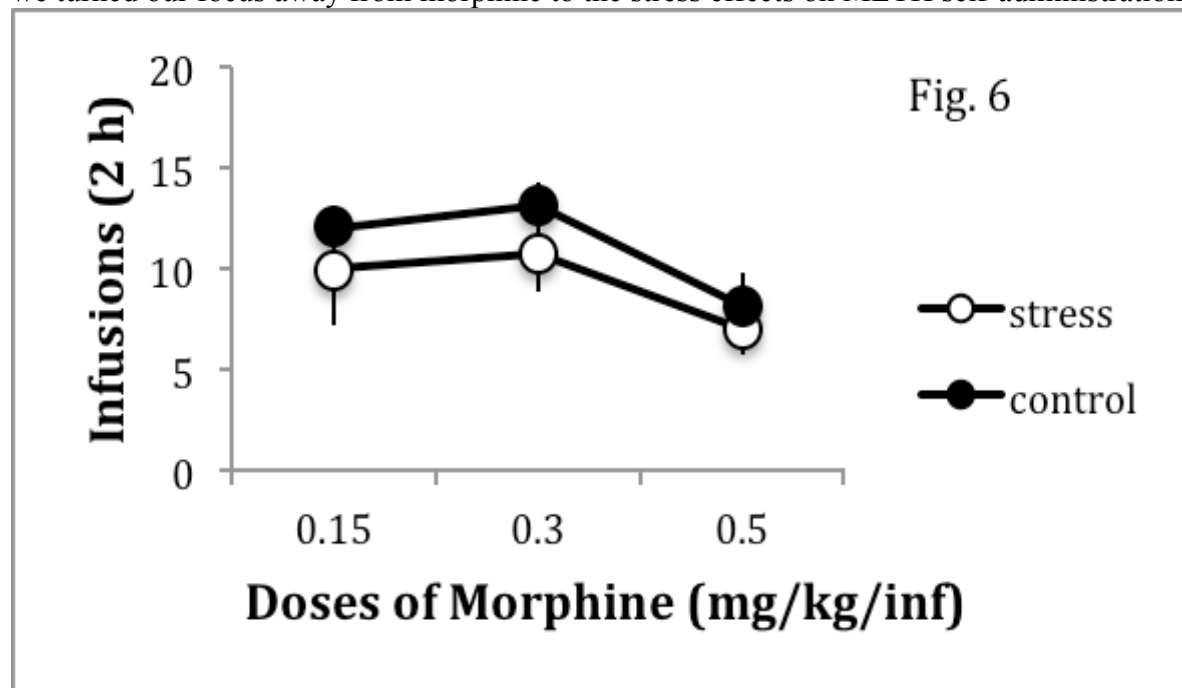
IIIA. Progressive ratio (PR) responding.

Concurrent with exploring mechanisms of stress-induced sensitization effects, we have been exploring the effects of stress on drug abuse behavior, which was proposed under Specific Aim III. The initial set of experiments examined whether prior exposure to acute stress would potentiate the acquisition, self-administration or reinstatement of morphine.

IIIA. Results

Morphine

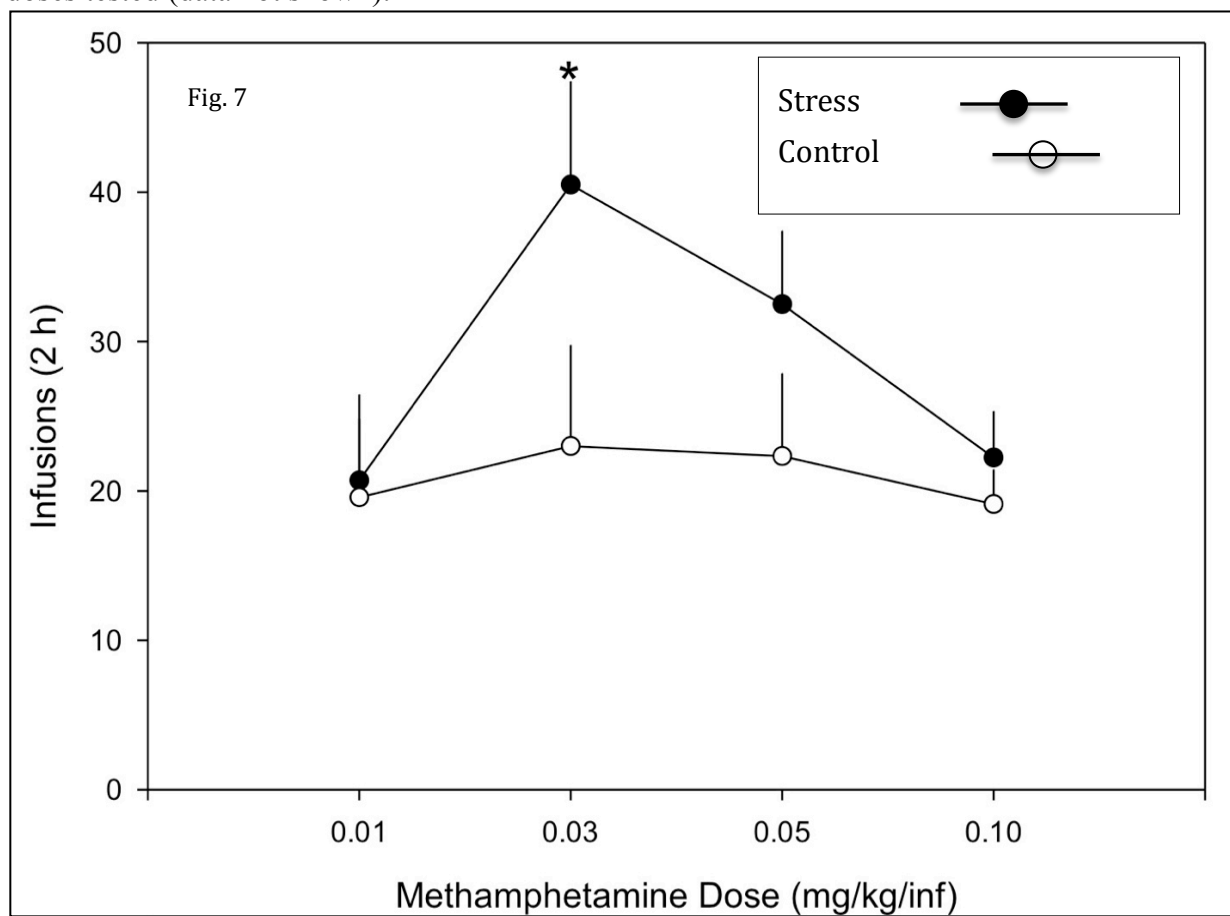
Animals were stabilized on a PR schedule at (0.75 mg/kg/injection for 1 wk prior to stress exposure) or served as HCC. 24 hr post-stress, animals were subject to a PR test (FR5). Fig. 6 shows the effects of stress on morphine self-administration (0.15, 0.3, and 0.5 mg/kg/infusion). Prior exposure to stress failed to modulate self-administration of morphine. Further, prior stress exposure had no effect on morphine reinstatement behavior. In light of the minimal effects of stress on the pro-inflammatory effects of morphine as well as self-administration of morphine, we turned our focus away from morphine to the stress effects on METH self-administration.



METH

METH Self-Administration and Reinstatement

Rats were exposed to inescapable tailshock or served as non-stressed controls (same parameters as in the previous experiments with morphine). Approximately 1 week post-stress, rats were trained to self-administer METH (0.1 mg/kg/infusion) in daily 2-h sessions, first on FR1 and then switched to FR5. Once stable self-administration behavior was maintained, rats self-administered 0.01, 0.03, 0.05 and 0.1 mg/kg/infusion (2 sessions at each dose; doses were counterbalanced). Fig. 7 shows that stressed and non-stressed rats both acquired self-administration of METH, and stress increased METH self-administration, particularly at the 0.03 mg/kg/inf dose (significant at 0.03; trend towards significance at 0.05). A follow-up experiment examined the effect of inescapable stress on the reinstatement of METH seeking, rats were first trained to self-administer METH (0.1 mg/kg/inf). Once stable self-administration behavior was maintained, the METH-reinforced behavior was extinguished. Following 5 days of extinction (4 hours/day), rats were exposed to inescapable shock or served as non-stressed controls. Rats were tested for the reinstatement of METH seeking 1 week after the stress session using METH (0, 0.3, 0.5 and 1.0 mg/kg, i.p.). Stress did not affect the reinstatement of METH seeking at the doses tested (data not shown).



Key Research Accomplishments

- At the protein level, acute stress potentiated the pro-inflammatory cytokine response to METH in key brain reward nuclei.
- METH failed to directly induce a pro-inflammatory response in isolated microglia.
- METH induced the alarmin HMGB1 in striatum.
- Acute stress potentiated METH self-administration.

Reportable outcomes

N/A

Conclusions

The present findings clearly show that stress sensitizes the neuroinflammatory response to METH and potentiates drug abuse behavior. The effects of stress and METH on the alarmin HMGB1 suggest that this danger signal may be induced as a result of METH neurotoxicity and thus may mediate the pro-inflammatory effects of METH as well as stress-induced neuroinflammatory priming effects.

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